Virulence Differences in Hypervirulent Klebsiella pneumoniae between Isolates with Multi-Locus Sequence Type (MLST)-11 and Serotype K1 or K2 Strains

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Research

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Abstract

Background: Two different types of hypervirulent *K. pneumoniae* (HvKp), including MLST-11 or serotype K1/K2 strains, have been frequently described in recent studies. Although these two types of strains were described as HvKp, their virulence was not compared. In this study, an *in vitro* and *in vivo* approach was used to assess differences in virulence.

Materials and Methods: A total of nine isolates, including one strain of serotype K1 isolate, two strains of serotype K2 isolates and six strains of ST11 isolates, were selected for this study. Phenotypic tests of virulence and associated genes were performed by string test, PCR, and *in vitro* models of serum resistance and phagocytosis.

Results: Although serotype K2 strains and ST11 isolates had similar virulence gene profiles, the ST11 isolates showed less serum and phagocytic resistance than did serotype K1/K2 isolates. The mouse lethality test revealed that all 11 ST isolates were unable to cause lethality, even with > 10^7 CFU, while one serotype K1 and two serotype K2 showed an LD_{50} of £ 10^2 CFU. Aerobactin (or capsule knockout mutants) exhibited a decline in LD_{50} compared to the parental strain, while capsule mutants showed a more significant decrease in LD_{50}.

Conclusion: Since there was a significant difference in virulence levels between the two types of HvKp when assessed by *in vitro* and *in vivo* animal models, the designation "HvKp" may be a better term based on animal studies and to avoid confusion. Virulence and nonvirulence could be analysed in a relative manner, especially in comparison studies.

Background

Klebsiella pneumoniae is a gram-negative bacterium and an opportunistic pathogen that can cause both community-acquired or nosocomial infection. Recently, *K. pneumoniae* has been categorized into classic *K. pneumoniae* (cKp) and hypervirulent *K. pneumoniae* (HvKp) due to differences in virulence. HvKp has been differentiated from cKp by its ability to develop the subsequent metastatic spread or to present in multiple sites of infections[1]. Metastasis, such as liver abscess in patients with meningitis or endophthalmitis [2–4], has been well described previously. Generally, cKp are often found and described from nosocomial isolates, while HvKp has been initially described in patients with community-acquired liver abscesses from Taiwan, Singapore, Hong Kong [5] and South Korea [6]. Subsequently, the term HvKp was recently presented in nosocomial isolates with a common multi-locus sequencing type (MLST) 11[2, 7]. This ST11 isolate was first identified to contribute to a significant outbreak with carbapenem resistance in the local hospital [7]. Alarmingly, this HvKp is multidrug resistant (MDR) and exhibits resistance to such drugs as carbapenems. This MDR-HvKp is difficult to treat [3], and pneumonia patients infected with this type of strain may die due to multiorgan failure or septic shock [2, 7]. MDR-HvKp encodes virulence genes, including several siderophore genes (*entB*, *iroN*, *iucA*, and *iutA*) and two capsular polysaccharide regulator genes (*rmpA* and *rmpA2*) that contribute to capsule expression. In

addition, this MDR-HvKp carried bla_{kpc2} genes for carbapenem-resistant pathotypes, which is in keeping with other classes of drug resistance. Apart from MDR-HvKp, another type of HvKp has also been frequently mentioned in the literature[8]. The clinical characteristics of infection are different. This type of HvKp is mostly derived from community-acquired infection, especially in liver abscesses and pneumonia. Diabetes is the only human risk factor for liver abscess patients with this type of HvKp infection. Although some virulence factors from liver abscess isolates are the same as from MDR-HvKp, the serotype prevalence is unique. Over 70–80% of isolates from liver abscesses are from serotypes K1 and K2. To the best of our knowledge, patients with complications of endophthalmitis or meningitis are all from serotype K1 or K2 isolates[9–11], while HvKp with ST11 are usually serotype K47 or K20 [7, 12, 13]. Notably, these two types of *K. pneumoniae* are all named HvKp in the literature (Table 1). However, there is no study comparing the virulence of these two types of HvKp. Thus, it may be difficult to define the term hypervirulence. In the present study, we compared these two types of strains named HvKp and investigated whether there is any difference in virulence that could be used to redefine hypervirulence.

Table 1
Previous publications used the term "hypervirulent *Klebsiella pneumoniae*" in the past 3 years by Medline searching

Year	Hypervirulent *K. pneumoniae*		
	[1]None	[2]Serotype K1 or K2	[3]ST11
2019	62	19	6
2018	70	22	10
2017	36	18	7
[1]Number of publications searched by Medline with the term "Hypervirulent *K. pneumoniae*" only.			
[2] Number of publications searched by Medline with the term "Hypervirulent *K. pneumoniae*" with serotype K1 or K2			
[3] Number of publications searched by Medline with the term "Hypervirulent *K. pneumoniae*" with ST11			

Methods And Materials

Serotyping and MLST for *K. pneumoniae* isolates

Isolates were collected from a previous study [14]. Serotyping K1 and K2 was performed by a rapid testing cassette and PCR [15, 16]. For nonserotype K1/K2 from rapid testing, serotyping was performed by using *wzi* and *wzc* sequencing [17, 18] and capsule-specific primers for serotyping [19]. MLST with the seven housekeeping genes (*gapA, infB, mdh, pgi, rpoB, phoE* and *tonB*) was performed via PCR using the corresponding primer pairs [20]. A total of 9 isolates were selected for this study.

Virulent gene profiling and the string test

Seven genes, *entB, iroN, iucA, iutA, clbA, rmpA* and *rmpA2*, were selected for detection of the *K. pneumoniae* isolate genes. The primers used for these virulence genes are listed in Supplementary Table 1 [2, 21, 22]. Bacterial DNA was prepared by suspending one loop of fresh colonies in 50 µl of DNAzol (DNAzol® DIRECT, Molecular Research Center, Inc. Cincinnati, OH) and heating the mixture at 95°C for 10 min. AmaR OnePCR (amaR OnePCR, GeneDireX®, Vegas, NV) was used as the PCR mixture, and the amplification procedure was followed by the manufacturer's protocol.

The string test was performed as a phenotypic method to assess virulence [4]. Isolates were streaked on a blood agar plate and kept to culture overnight. Criteria of hypermucoviscosity were defined as previously described [7, 11, 23]. The colony was stretched with an inoculation loop to measure the visible string, and more than 5 mm was considered a hypermucoviscosity phenotype.

Generate aerobactin and capsule knockout mutants by in-frame deletion

In-frame deletion mutagenesis was used to generate mutants for virulence studies [24]. In brief, the primer sets iucA-AR and iucA-BF were used for *iucA* deletion construction (Supplementary Table 2), and these primer sets were complementary to each other. The PCR fragment was digested with XbaI and SacI and then cloned into the puTkmy-MCS plasmid for 799 and 794 strains and the puTkmy-MCS-zeocin plasmid for 3016 strain. These plasmids are suicide vectors harbouring the *E.coli* sacB gene. When this gene is expressed on the plasmid, a sucrose-sensitive phenotype was present to enable positive selection with sucrose and detect the loss vector. The single-crossover strains were selected from green inositol-nitrate-deoxycholate (BIND) plates. The green plate was coated with kanamycin (50 µg/ml) for 799 and 794 strains and zeocin (25 µg/ml) to prevent the growth of non-Kp contaminants. The transconjugant was selected and verified with PCR via primer sets [25] (Supplementary Table 2).

In vitro virulence assessment by neutrophil phagocytosis and the serum resistance assay

A neutrophil phagocytosis assay was performed as previously described [26]. Isolation of neutrophils and pooled serum from healthy volunteers received ethics approved from the Research Ethics Committee of the National Health Research Institute (number: EC1061212-E)

The serum bactericidal assay was modified and described by Podschun's study and from our previous study [24, 27]. In brief, the bacteria were streaked on Mueller-Hinton agar (MHA) plates overnight to collect a single colony to embed in brain heart infusion (BHI) broth and measured with optical density at 600 nm (OD_{600}) to 0.4. After dilution with phosphate-buffered solution (PBS) from 10^8 to 10^6, the bacteria were combined with 750 µl of human serum and incubated for 0, 1, 2, and 3 hours. The mixture of each time frame was serially diluted, and 10^{-3}, 10^{-4}, and 10^{-5} were streaked on the MHA plate for visual bacterial counts.

Mouse lethality test

Male BALB/c mice aged 6-8 weeks (n=6/strain) were used in the lethality testing. They were purchased from the National Laboratory Animal Center, Taiwan, and housed in the National Defense Medical Center Laboratory Animal Center. The animal protocols of this study were reviewed and approved by the Institutional Animal Care and Use Committee of the National Defense Medical Center (IACUC-19-271) and National Health Research Institute (NHRI) (NHRI-IACUC-107009-A).

Strains were analysed by the acute lethality test in the mouse model. The day before the experiment, the bacteria were streaked on MHA plates and grown in the incubator overnight. The bacteria were transferred to BHI broth and kept in the incubator until OD_{600} of 0.9. After centrifugation and serial dilutions with PBS, bacteria were randomly intraperitoneally (i.p.) injection to the mice (0.1 ml/mouse, acute injection). Within 14 days of observation, the lethality and virulence of the bacterium were observed and detected by the mouse survival rate. Food and water were provided *ad libitum,* and cages with new bedding were changed once a week. This experiment was duplicated to confirm the virulence degree of the bacterium. The LD_{50} was calculated using SigmaPlot version 7.0 from SPSS Inc. (Chicago, IL).

Results

MLST, serotyping and virulence-associated genes of the isolates selected in this study

MLST of 9 isolates revealed that one of each isolate had ST65, -373, or -23. The remaining six isolates had ST11 (Table 2). Serotyping by rapid test cassette and PCR showed that 3 isolates belonged to serotype K1 or K2. Two serotype K2 and one serotype K1 isolates had ST65, -373, and – 23, respectively. The remaining 6 isolates with ST11 were non-K1/K2 isolates. Furthermore, PCR *wzi* and *wzc* sequencing showed that non-K1 and K2 isolates had *wzi* and *wzc* sequences close to serotype K20. PCR confirmation by serotype-specific primers of K20 showed that all six isolates were serotype K20 (Table 2). All genotype K1, K2 or ST11 isolates had been determined to have the same virulence-associated genes, *entB*, *iroN*, *iucA*, *iutA*, *rmpA and rmpA2*, except for the strains of NVT1001 and 794 with serotypes K1 and K2, respectively, carrying one additional virulence-associated gene, *clb*. Thus, a total of 3 different serotypes of isolates with four different MLST were included in further assessment of virulence by *in vitro* and *in vivo* models (Table 2).

Table 2
Serotype, MLST and virulence-associated gene detection among the isolates in this study

Isolate no.	Serotype	MLST type	Virulent genes						
			clb	*entB*	*iroN*	*iucA*	*iutA*	*rmpA*	*rmpA2*
NVT 1001S	K1	23	+	+	+	+	+	+	+
794	K2	65	+	+	+	+	+	+	+
799	K2	373	-	+	+	+	+	+	+
3016	K20	11	-	+	+	+	+	+	+
3386	K20	11	-	+	+	+	+	+	+
3423	K20	11	-	+	+	+	+	+	+
3458	K20	11	-	+	+	+	+	+	+
3500	K20	11	-	+	+	+	+	+	+
3502	K20	11	-	+	+	+	+	+	+

String test of serotype K1, K2 and ST11 isolates and their derived mutants.

Since iron acquisition-related systems or capsule production-related systems have been described as the major factors contributing to the hypervirulence and the string test was suggested as a means of rapidly detecting of the virulence degree of unknown isolates [11, 23], we performed string testing as the initial assessment of virulence. Our results indicated that parental serotype K1/K2 isolates and their iron acquisition-related *iucA* mutants were positive (more than 5 mm) to initial screening by the string test. The string test indicated that *iucA* mutants did not contribute to hypermucoviscosity, while *cps* mutants showed loss of mucoviscosity. Isolates with ST-11, including parents and mutants, were negative for the string test (Fig. 1).

Neutrophil phagocytosis, serum resistance and mouse lethality between serotype K1/K2 and ST11 isolates

Neutrophil phagocytosis with human serum opsonization was used to assess the bacterial response to the first-line human defence mechanism. Among parental isolates of serotypes K1, K2 and ST11, with adjusted zero at 0 min, serotypes K1 and K2 were relatively more resistant to phagocytosis than the ST11 isolate (Fig. 2). Compared to their *iucA*, *cps* and *iucA*/*cps* double mutants, all *cps* mutants were more susceptible to phagocytosis (Fig. 3A-D). Although aerobactin (*iuc*) was suggested to be an important factor contributing to virulence, Δ*iucA* mutants express phagocytic resistance similar to that of their parental strains. The results obtained from phagocytosis indicated that *iuc* and *cps* play different roles in virulence. CPS played an important role in the resistance to neutrophil phagocytosis, while *iuc* did not play a role in neutrophil phagocytosis.

For the serum resistance test, pooled normal human serum was collected from 10 healthy adults to examine the complement killing effect on K1/K2 and ST11 isolates. Serotype K1/K2 strains and Δ*iucA* mutants showed more resistance (grade 5–6) to serum complement killing. CPS mutants or double mutants of Δ*iucA*/Δ*cps* showed various susceptibilities to serum complement killing. The Δ*cps* and Δ*iucA*/Δ*cps* of NVT100 and 794, respectively, from serotypes K1 and K2 remained resistant to complement killing (grade 5–6), while strain 799 with serotype K2 showed a significantly increased susceptibility and became susceptible to complement killing (grade 1). Clinical isolates of ST11 and their mutants were susceptible (grade 1) to serum complement killing. Since all 6 parental ST11 isolates were serum susceptible, the serum susceptibility of Δ*cps* and Δ*iucA* mutant were not determined (Table 3).

Table 3
Summary of the phenotypic and virulence differences among the hypervirulent *K. pneumoniae* and their mutants in this study

Isolation no.	Serotype	MLST	Strain	Serum assay (Grade 1–6)	String test (≥ 5 mm)	LD_{50}
NVT1001S	K1	ST23	wt	R (6)	+	3.98×10^2
			Δ*iucA*	R (6)	+	1.0×10^4
			Δ*cps*	R (6)	-ve	$\geq 10^7$
			Δ*iucA*Δ*cps*	R (6)	-ve	$\geq 10^7$
794	K2	ST65	wt	R (6)	+	2.4×10^2
			Δ*iucA*	R (5)	+	4.17×10^4
			Δ*cps*	R (6)	-ve	$\geq 10^7$
			Δ*iucA*Δ*cps*	R (6)	-ve	$\geq 10^7$
799	K2	ST373	wt	R (6)	+	$\leq 10^2$
			Δ*iucA*	R (6)	+	$\leq 10^2$
			Δ*cps*	S (1)	-ve	$\geq 10^7$
			Δ*iucA*Δ*cps*	S (1)	-ve	$\geq 10^7$
3016	K20	ST11	wt	S (1)	-ve	$\geq 10^7$
			Δ*iucA*	S (1)	-	$\geq 10^7$
			Δ*cps*	S (1)	-	$\geq 10^7$
			Δ*iucA*Δ*cps*	S (1)	-	$\geq 10^7$
3386	K20	ST11	wt	S (1)	-ve	$\geq 10^7$
3423	K20	ST11	wt	S (1)	-ve	$\geq 10^7$
3458	K20	ST11	wt	S (1)	-ve	$\geq 10^7$
3500	K20	ST11	wt	S (1)	-ve	$\geq 10^7$
3502	K20	ST11	wt	S (1)	-ve	$\geq 10^7$

In vivo assessment of virulence was examined in mice for 14 days via intraperitoneal (IP) injection with different inoculums of *K. pneumoniae* isolates and their derived mutants. Mice that received IP injections of K1 (1001) and K2 (799 and 794) isolates showed significant mortality compared to ST11 isolates beginning on day 5 post-injection (Fig. 4A, B), while the Δ*iucA* or capsule knockout mutants showed a different degree of decreased lethal dose in mice. The virulence gene knockout groups, including *cps* and *iucA/cps* mutants, all survived with high CFU injection (Fig. 4C), indicating a major virulence contribution of *cps* mutants. These results demonstrate that K1 and K2 were significantly more virulent and that relatively low concentrations of bacteria were sufficient to kill a significant number of the animals in the groups. The mice that received ST11 isolate injections survived without symptoms of illness after 14 days with high concentrations ($> 10^7$ CFU) (Fig. 4D). In addition, the virulence degree of ST11 isolates was comparable with *cps* mutants and Δ*iucA/*Δ*cps* mutants, and mice in both groups survived with no symptoms of infection. ST11 isolates might carry similar degrees of virulence compared to virulence factor mutants, implying that the ST11 isolates in this study are avirulent. In summary, in the assessment of virulence by different models (Table 3), ST11 was less virulent in both the *in vitro* and *in vivo* models. The ST11 isolates were less susceptible to neutrophil phagocytosis and serum resistance, and no mouse lethality was observed, even at high doses of inoculums (10^7 colony forming units). Table 3 summarizes the phenotypic and virulence differences among the HvKp strains and their mutants for the factor contributing to virulence. Although all parental strains, K1, K2 and ST11, were termed HvKp according to a previous publication, the virulence varied in different assessment models.

Discussion

The term HvKp has been frequently described in recent publications. Many studies using this term are based on patients with severe infectious disease, such as the development of complications or mortality [1, 2, 4]. Through the collection of isolates from these patients, experimental work was performed on *K. pneumoniae* to determine the possible cause of the severe illness. Aside from patient factors, invasiveness or virulence is thus considered an important bacterial factor affecting severity or worse clinical outcome [2, 4]. By comparing the isolates with the cause of severe illness and without cause of severe diseases, isolates with the virulence difference that could be identified by any virulence assessing models and the contributing factor/s of virulence difference that could be distinguished will be considered to be used for the term HvKp. In this study, we investigated isolates that were termed HvKp according to previously published descriptions and assessed these isolates under the same experimental platform. Serotypes K1/K2 and ST11 are the two groups that are frequently described as HvKp in recent publications (Table 1). Under the determination of virulence factors that have been described previously, we selected isolates with similar virulence gene profiles in this study to minimize the virulence degree difference caused by these two groups of strains (Table 2). Except for *clbA*, one of each serotype K1 and K2 contained an extra *clbA* (colibactin), and all other isolates, including one K2 isolate and 6 ST11 isolates, have virulence gene profiles that are identical to those of *entB, iroN, iucA, iutA, rmpA* and *rmpA2*.

The string test has been widely used as an initial screening of virulent *K. pneumoniae*. Both serotypes K1/K2 were positive to string, while all ST11 isolates were negative. These results were in keeping with the previous studies finding that serotype K1/K2 isolates were generally more mucoid than the other serotype [4, 9, 19, 28]. The Δcps of serotype K1/K2 lost the mucoid phenotype, indicating that cps is involved in the string test results. Since cps is highly correlated with neutrophil phagocytic resistance [26], serotype K1/K2 isolates with a higher resistance to phagocytosis than ST11 were not unexpected. One intriguing finding is that cps knockout mutants have shown various levels of serum resistance between two serotype K2 isolates. Previous studies have shown that serum resistance varies. Simoons-Smit et al. [29] observed that the loss of K antigen (capsule) could enhance serum-mediated complement killing, while Tomás et al. found that the capsular polysaccharide seemed not to play any important role in resistance to serum bactericidal activity in this bacterium [30]. The varied serum resistance of different cps knockout strains has also been identified in our previous study, indicating that a cofactor with cps may contribute to the variation in serum resistance in cps knockout strains [16]. The inconsistencies among studies warrant further investigation.

In the mouse lethality test, the mean range of survival rates was between 10^2 and $\geq 10^7$ CFU. Our previous criterion to assess virulence was LD_{50}, with $\leq 10^2$ CFU representing hypervirulence and $\geq 10^7$ indicating avirulence. The results in this study have shown that there is a large difference in the lethal dose to mice among these HvKp isolates. K1/K2 isolates have an LD_{50} between $\leq 10^2$ and 10^3 CFU, while mice have shown no symptoms of illness when challenged with K20 ST11 isolates with a 10^7 CFU, a saturated dose for IP injection. Since one serotype K2 isolate, 799, has an identical virulence gene profile as ST11 isolates, the extra *elbA* could not explain the large difference in virulence between serotype K1/K2 and ST11 isolates.

Aerobactin has been suggested to play an important role in virulence and is also a key biomarker in enhancing the growth and survival of HvKp [23, 31]. Our results demonstrated that the aerobactin mutant from serotype K1/K2 parental isolates decreased LD_{50} by 100-fold in mice lethality (Table 3 and Fig. 4). The virulence degree could drop from hypervirulence to virulence according to the criterion of virulence categorization [4]. Thus, aerobactin plays an important role in virulence and should be considered in patients infected with aerobactin carrying *K. pneumoniae*. In this study, the contribution of virulence between capsular polysaccharides with specific serotypes and aerobactin was compared. In the mouse lethality test, *cps* is a major factor contributing to the degree of virulence. Loss of the capsule will cause a substantial decline in virulence from HvKp to avirulence (Fig. 4).

Previous studies used wax moth *Galleria mellonella* larvae to assess the virulence of the Kp pathogen. Since moth larvae have a short life span and are easy to reproduce in the laboratory environment, quantitative studies suggest using moth larvae as an *in vivo* model [2, 32]. However, a comparative study in ST258 strains described different degrees of virulence in mammals and nonvertebrates, and rapid death in moth larvae was avirulent in the mice [33]. More studies have used mice to investigate human infectious disease and to examine the lethality and toxicity response in rodents, and the results are

reliable [23, 24, 34]. One clinical study from China investigated unknown Kp isolates collected from a local hospital. With a mouse lethality test and serum assay, only the K2 isolate was virulent ($< 10^2$), and ST11 isolates were nonvirulent ($> 10^6$) [8]. The result corresponded to our present study.

To assess the virulence of bacteria, both *in vitro* and *in vivo* models can be adopted. The selection of models depends on the factor being assessed and the reference that is used for the comparison. The choice of model becomes an important point to differentiate from one strain to another. Different models will have limitations due to the magnitude of the factor that can be assessed or the window of the factor that can be magnified enough to determine the difference. Thus, the choice of an infection model may lead to over- or underestimation of the interpretation of virulence. In this regard, the term HvKp is used in two different types of *K. pneumoniae.* However, to determine causes of human infection, *in vivo* or animal models are better accepted as closed models than are *in vitro* models to assess the level of virulence in humans.

Previous studies on HvKp have adopted different *in vivo* models to assess extreme virulence, but those studies were studied individually. Individual studies of HvKp were not problematic. Those works showed a higher level of virulence than the classic reference *K. pneumoniae*. After these two different types of HvKp were placed into the same animal model, the level of virulence was differentiated. In conclusion, although HvKp has been described frequently in different studies, the virulence was notably different when assessed in the same animal model. Since animals are closer to reflecting human conditions, we suggest that the term hypervirulence be based on animal models instead of others to avoid confusion with virulence against *K. pneumoniae*. Virulence and non-virulence could be used in a relative manner, especially in comparison studies.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

All authors read and approved the final manuscript.

Availability of data and material

The raw data involved in study could be provided upon request. Bacteria strains will be provide for academic use only and transferring has to be approved by safety department.

Competing interests

The authors declared that they have no competing interests.

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Authors' contributions

TCW, JCL, FYC and LKS designed the study and drafted the manuscript. TCW, YWH, SKC and CHW carried out the experiments. CPF, FYC and LKS proof read and edited the manuscript.

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Figures

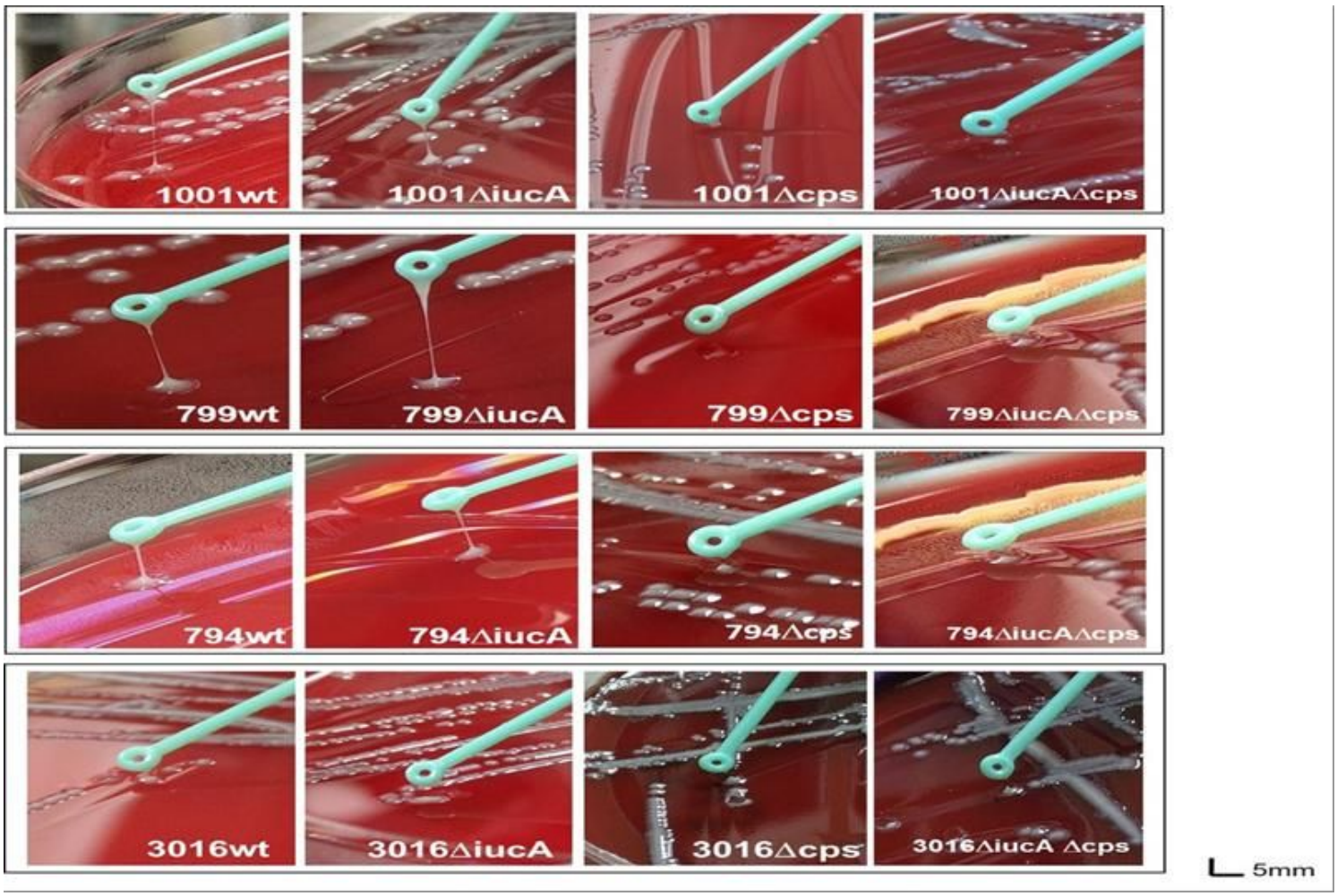

Figure 1

Phenotypic virulence assessment by string test on wild-type HvKp isolates and their derived mutants.

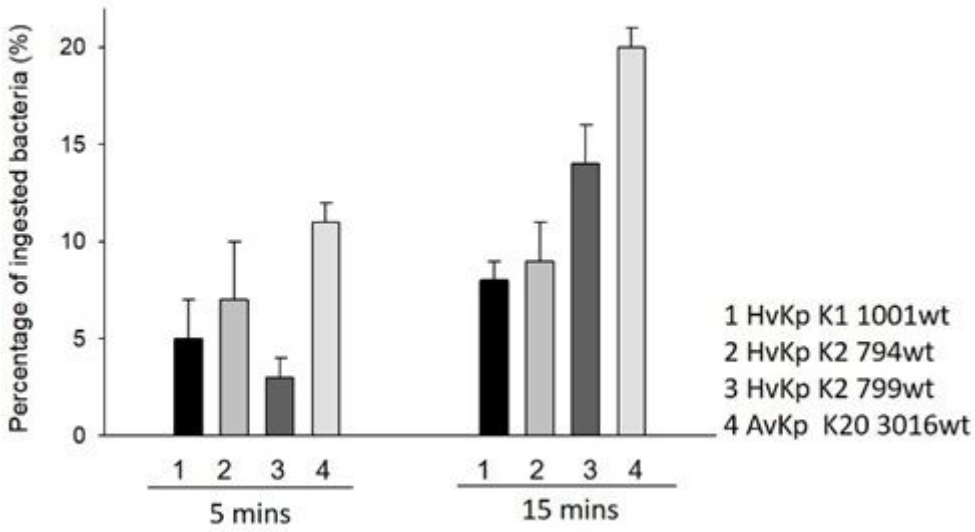

Figure 2

Neutrophil phagocytosis among HvKp from wild-type serotype K1/K2 and ST11 isolates.

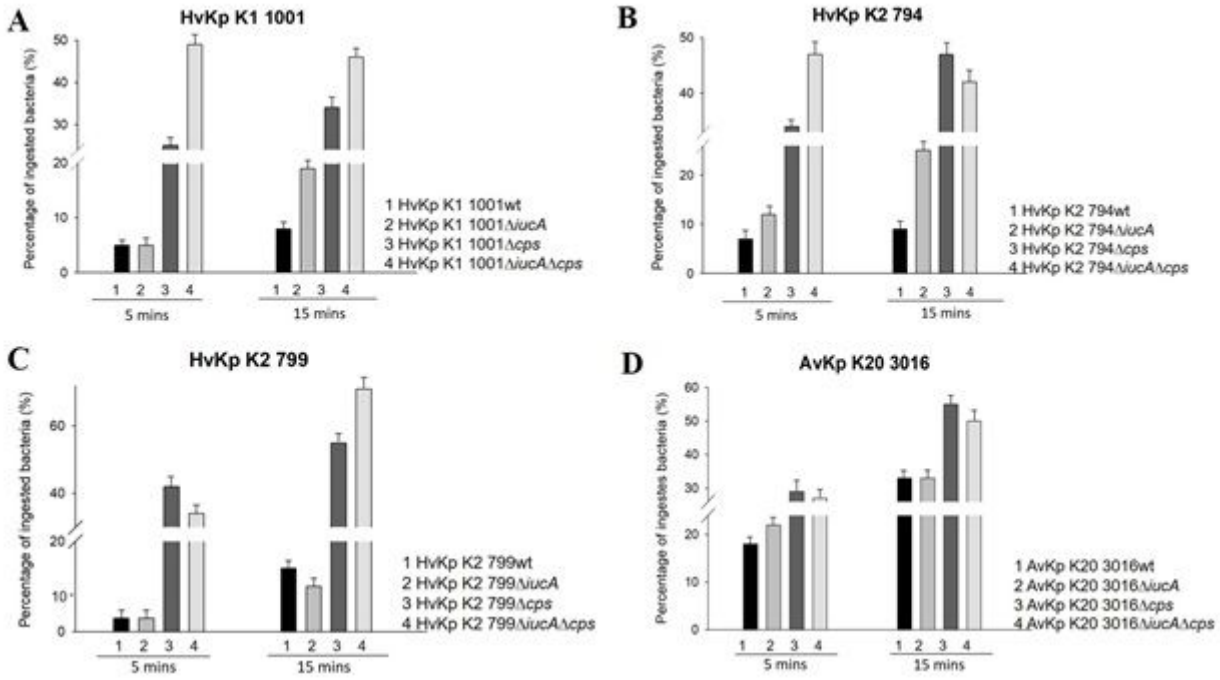

Figure 3

Neutrophil phagocytosis among HvKp-derived mutants from wild-type isolates. K1/K2 and ST11 isolates. Mutants included iucA, cps and iucA/cps double mutants.

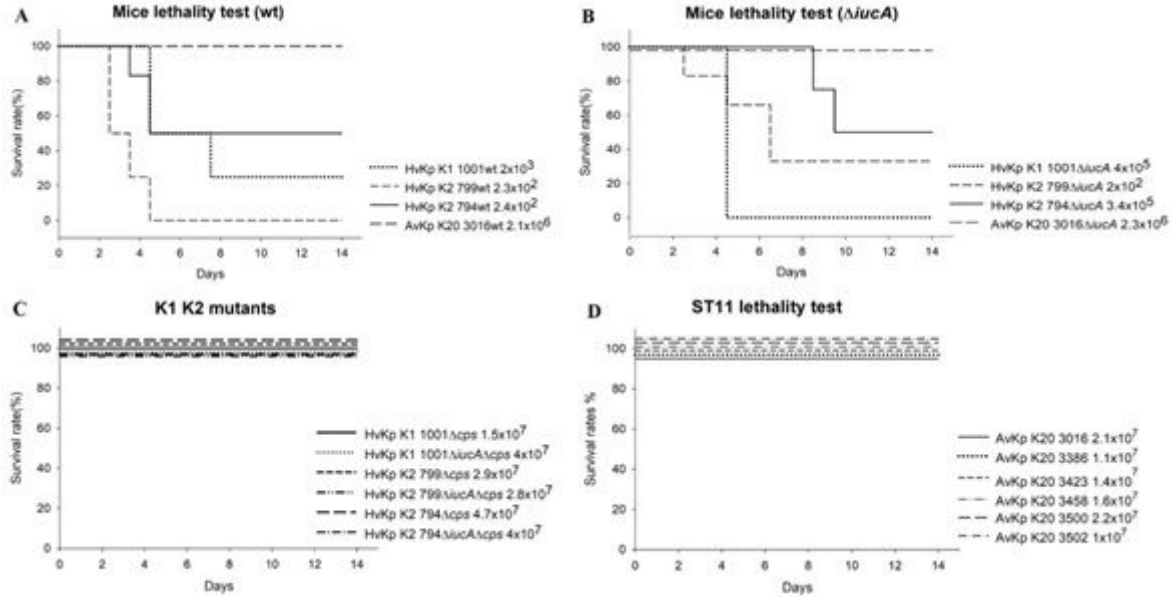

Figure 4

Mice lethality, LD50, wild-type HvKp isolates and their derived mutants.